THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

V. ON THE CHEMICAL NATURE OF THE ALDOBIONIC ACID FROM THE SPECIFIC POLYSACCHARIDE OF TYPE III PNEUMOCOCCUS.

By MICHAEL HEIDELBERGER and WALTHER F. GOEBEL.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, June 25, 1927.)

The so called soluble specific substances of the three fixed types of Pneumococcus appear identical with three chemically distinct, serologically type specific polysaccharides produced by these organisms during growth in culture media (1). The immunological significance and the chemical nature of these bacterial carbohydrates have been discussed in detail in earlier publications from this laboratory. The present communication deals with the chemical nature of an aldobionic acid, the fundamental building stone of the polysaccharide derived from Type III pneumococcus.

This aldobionic acid, the product of hydrolysis of the Type III specific carbohydrate, has been shown (2) to have the formula $C_{11}H_{19}O_{10}COOH$ and to be built up from a hexose (glucose) and a hexose-uronic acid of unknown nature, in such a manner that the carboxyl group and one aldehydic group remain free. It is of interest to extend the investigation of this substance, for it not only appears unique in the field of sugar chemistry, but important in that it or its isomers are found among the hydrolytic products of specific carbohydrates from other microorganisms. The present report aims to identify the hexose-uronic acid which forms half of the molecule of the aldobionic acid, and to explain the nature of the glucosidic linkage which binds the sugar to the acid.

EXPERIMENTAL.

1. Preparation of the Aldobionic Acid.

30 gm. of air-dry specific polysaccharide (prepared as in Paper IV (2)) were dissolved in 120 cc. of 75 per cent sulfuric acid (by weight) at 0°. After standing overnight in the ice box the solution was diluted to 3 liters and boiled 5 hours under reflux. The sulfuric acid was then quantitatively removed with highly purified barium hydroxide and the barium sulfate washed free from reducing sugars. The combined filtrates were concentrated to 200 cc. *in vacuo*, boiled with a little norit and an excess of calcium carbonate, filtered, and the filtrate concentrated to 100 cc. *in vacuo*. The solution, which contained small amounts of glucose and the calcium salt of the aldobionic acid, was poured into 10 volumes of methyl alcohol. In this manner the crude calcium aldobionate was freed from reducing sugars. The suspension was filtered and washed two or three times with methyl alcohol. 24 gm. of crude calcium aldobionate were thus obtained.

2. Preparation of Pure Calcium Aldobionate.

The crude salt was dissolved in twice its weight of water, and to the solution was added alcohol in small portions. After each addition of alcohol the mixture was centrifuged. Enough alcohol was added so that after the final centrifugation the supernatant liquid, still containing a large part of the original calcium salt, remained as a pale straw-colored solution. This was decanted from the deeply colored lower oily layer and saved. The lower layer was again dissolved in an equal volume of water, and treated as before with alcohol, the straw-colored supernatant liquid again being saved. After fractionating the lower layer two or three times more, a deeply colored oil was obtained which was discarded, and the combined supernatant liquids were concentrated *in vacuo* and poured into 10 volumes of methyl alcohol. 18 gm. of purified calcium aldobionate were finally isolated. The substance gave the following analysis:

0.1028 gm. substance: 0.1438 gm. CO_2 and 0.0490 gm. H_2O.
0.1096 " " : 0.0092 " CaO.

Calculated for $(C_{12}H_{19}O_{12})_2Ca$. C 38.33 per cent, H 5.07 per cent, Ca 5.33 per cent. Found. C 38.14 per cent, H 5.33 per cent, Ca 6.00 per cent.

The free aldobionic acid was obtained by adding a little less than the calculated amount of oxalic acid to a 5 per cent solution of the calcium salt, filtering off the calcium oxalate, concentrating the filtrate to dryness *in vacuo*, and dissolving the residue in methyl alcohol. The alcoholic solution of the aldobionic acid was filtered from the small amount of insoluble calcium aldobionate, and the filtrate was evaporated to dryness *in vacuo*. The sugar, if properly manipulated, will puff up into a spongy mass, and when completely dry it may be readily broken up and scraped from the flask.

3. Oxidation of the Aldobionic Acid with Barium Hypoiodite.

5.0 gm. of aldobionic acid were dissolved in a small amount of water and the solution was oxidized with barium hypoiodite by a method previously described (3). The solution of the oxidation product, after being freed completely from inorganic constituents, was boiled with calcium carbonate and a small amount of norit. It was then filtered and concentrated to 30 cc. *in vacuo*. By the gradual addition of alcohol a precipitate of the calcium salt of the oxidized aldobionic acid, which will be termed glucurono-gluconic acid, separated out. This calcium salt is far less soluble than is that of the aldobionic acid, 25 cc. of alcohol sufficing to remove it from solution. The calcium salt was filtered off and weighed. 5.2 gm. were recovered.

0.1000 gm. substance: 0.1284 gm. CO_2 and 0.0426 gm. H_2O.
0.1368 " " : 0.0188 " CaO.
Calculated for $C_{10}H_{18}O_9(COO)_2Ca$. C 35.12 per cent, H 4.42 per cent, Ca 9.76 per cent. Found. C 35.01 per cent, H 4.77 per cent, Ca 9.82 per cent.

4. Properties of the Glucuronogluconic Acid.

This dicarboxylated acid, when free from calcium, forms a white amorphous powder soluble in methyl alcohol, and less soluble in ethyl alcohol. It is soluble in hot glacial acetic acid, and, on cooling, droplets of oil separate which do not crystallize on standing. The free carboxylated sugar is a strong acid, and an aqueous solution colors Congo red paper a vivid blue. The calcium salt shows $[\alpha]_D = -7.5°$.

The acid itself gives a strong naphthoresorcinol test and on distillation with 12 per cent hydrochloric acid it yields 17 per cent

of furfural. The acid is non-reducing, but when boiled with strong hydrochloric acid reducing sugars appear. On hydrolyzing for 15 hours with normal hydrochloric acid it yields a maximum of 24.8 per cent reducing sugars (calculated as glucose). It was thought possible to hydrolyze the acid with sodium amalgam as Levene and La Forge (4) hydrolyzed chondrosin, but experiments in this direction failed. The acid is extremely stable to hydrolysis, both by acids and alkalies.

5. Hydrolysis of the Aldobionic Acid with Bromine and Hydrobromic Acid.

2.0 gm. of aldobionic acid were dissolved in 50 cc. of N hydrobromic acid and to the solution was added 0.5 cc. of bromine. The mixture was boiled under a reflux for 20 hours. It was necessary to replace the bromine from time to time. At the end of the hydrolysis the solution gave only a very faint naphthoresorcinol test and showed no reduction. The solution was then evaporated *in vacuo* to remove hydrobromic acid and bromine. The remaining traces of acid were removed with silver sulfate and the silver bromide was filtered off. The silver ion in the filtrate was removed with hydrogen sulfide and after filtration the sulfate ion was removed quantitatively with barium hydroxide. A filtrate free from inorganic constituents was thus obtained. This filtrate was evaporated to 2 cc. *in vacuo*, made strongly alkaline with 50 per cent potassium hydroxide, and then acidified with glacial acetic acid. The solution was seeded with a small crystal of potassium acid saccharate and was placed in the ice box. After standing 24 hours crystals of potassium acid saccharate (0.20 gm.) were filtered from the mother liquor. The crude salt was recrystallized from 1 cc. of boiling water.

0.0474 gm. substance gave 0.0168 gm. K_2SO_4.

Calculated for $COOK(CHOH)_4COOH$,	K 15.75	per cent.
Found.	" 15.89	" "

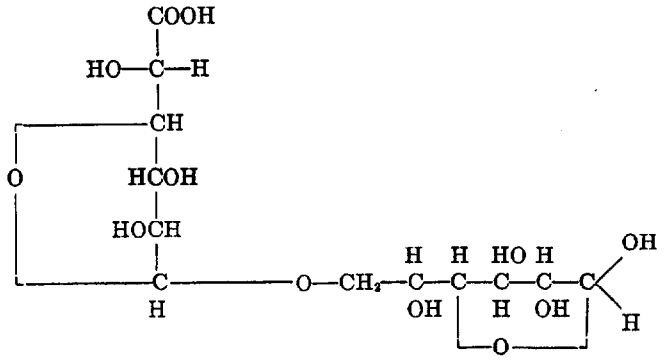
On substituting glucose for the aldobionic acid, a repetition of the above experiment gave no potassium acid saccharate.

DISCUSSION.

Since it had previously been shown that the aldobionic acid, the chief hydrolytic product of the specific polysaccharide of Type

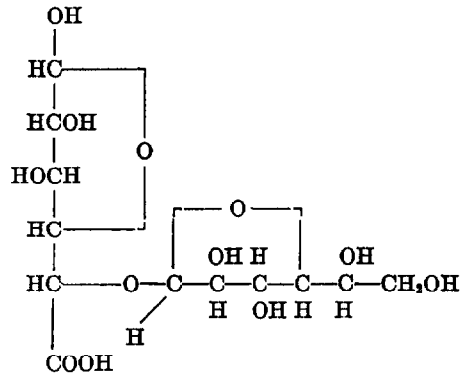
III pneumococcus, is composed of glucose and a hexose-uronic acid of unknown nature, united in such a manner that one aldehydic group remains reactive in the bionic acid molecule, it must necessarily be assumed that the sugar and sugar acid are combined in glucosidic linkage either through the aldehydic group of the glucose or through that of the hexose-uronic acid. If the linkage were of the first type, then the product obtained by oxidation of the free aldehydic group with barium hypoiodite would obviously be a true glucoside of a dibasic hexose acid; if the linkage were through the aldehydic group of the hexose-uronic acid, the latter would be intact after oxidation, whereas the free aldehydic group of the glucose would be oxidized. Since the glucuronogluconic acid, obtained by the oxidation of the aldobionic acid, still gives a naphthoresorcinol test and still yields the same amount of furfural on distillation as it did before oxidation, one must assume that the hexose-uronic acid does remain intact and that the free aldehydic group of the aldobionic acid is actually the reducing group of the glucose half of the molecule and not that of the uronic acid. It has been shown above, that the aldobionic acid yields saccharic acid on hydrolysis in the presence of bromine. Since glucose does not yield this acid under these conditions, one must necessarily assume that the saccharic acid formed in the above experiment owes its origin to the hexose-uronic acid part of the aldobionic acid molecule and that the hexose-uronic acid is therefore glucuronic acid.

The formula (I)



I.

is in accord with the results of the experiments which have been performed, whereas the isomeric formula (II)



II.

is not.

Whether the linkage between glucuronic acid and glucose is through carbon atom (6), in formula (I), or through one of the other carbon atoms, remains to be determined.

SUMMARY.

The aldobionic acid $C_{11}H_{19}O_{10}COOH$ isolated from the hydrolytic products of the specific polysaccharide of Type III pneumococcus has been shown to be a compound of glucuronic acid and glucose, combined in glucosidic linkage through the aldehydic group of glucuronic acid and one of the carbon atoms of glucose.

In conclusion the writers wish to express their gratitude to Dr. P. A. Levene for his many helpful suggestions, particularly his suggestions on the manipulation of sugar acids.

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